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Potential Probiotic Therapy for the Prevention of Antibiotic Associated Diarrhoea by using Lactobacillus Strain L9 Isolated from Soibum

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Abstract

Antibiotic associated diarhoea (AAD) is defined as an unexplained diarrhoea that occurs in association with the administration of antibiotics. Enteric pathogens like *Salmonella*, *Clostridium perfringens* type A, *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*, *Shigella flexneri*, *Vibrio cholera*e and *Enterobacter aerogenes* and possibly *Candida albicans* has been associated with antibiotic- induced diarrhea. Probiotics commonly administered in randomized controlled trials of AAD are: *Lactobacillus acidophilus, Lactobacillus bulgaris, Lactobacillus casei, Lactobacillus rhamnosus, Bifidobacteria bifidum, Bifidobacteria longum, Streptococcus thermophiles, Saccharomyces boulardii*. The isolate L9 from soibum was found to be effective against the enteropathogens. The efficacies of commercially available antibiotics for AAD were also found to be increased when incorporated along with the isolate. This technique of using the isolate along with the antibiotics could be useful in the treatment of antibiotic resistant pathogens.

Keywords

Antibiotic- associated diarrhea, Enteropathogens, *Lactobacillus,* Probiotic, Soibum

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INTRODUCTION

Antibiotic associated diarrhea (AAD) is defined as an unexplained diarrhoea that occurs in association with the administration of antibiotics (15). The World Health Organization defines AAD as three or more abnormally loose vowel movements per 24 hours. It is associated with altered intestinal microflora, mucosal integrity, vitamin/mineral metabolism and abdominal cramps (6). More than 400 species of bacteria inhabit the human gut and a balance of these microorganisms is important for normal gastrointestinal function (3). Antibiotic treatment may disturb the normal gut microflora resulting in a range of clinical symptoms most notably diarrhea. Enteric pathogens that can cause diarrhoea includes *Salmonella, Clostridium perfringes type A, Staphylococcus aureus, Escherichia coli, Staphylococcus aureus, Salmonella enterica, Shigella flexneri, Vibrio cholera* and *Enterobacter aerogenes* and possibly *Candida albicans* has been associated

with antibiotic- induced diarrhea (3). *Clostridium difficile* overgrowth is the bacterial agent most associated with AAD (10-20%) and occurs most often in older, immune compromised, hospitalized adults, but also occurs in children (2).

Drugs have multiple effects on the gastrointestinal tract (7), including some that are independent of anti-microbial activity. Erythromycin acts as a motilin receptor agonist and accelerates the rate of gastric emptying. The clavulanate in amoxicillin-clavulanate appears to stimulate small vowel motility, and in rare stances, penicillin may cause segmental colitis. Antibiotics may substantially reduce the concentration of fecal anaerobes that are normally present. As a consequence, the metabolism of carbohydrates may decrease which causes osmotic diarrhea. And the rate of breakdown of primary bile acids, which are potent colonic secretory agents, may be reduced. Neither mechanism is clearly established as a cause of AAD, but the efficacy of enemas with food flora in treating this problem suggests that changes in fecal flora are a contributing factor (8).

Probiotics are non-pathogenic bacteria or yeasts which are administered prophylactically or therapeutically in an attempt to modify the mucosal, epithelial, intestinal and systemic immune activity in ways that may benefit human health. The rationale behind probiotic administration is based on re-inoculation and normalization of unbalanced indigenous micro flora using these strains. Probiotics are reported to improve microbial balance in the intestinal tract and display both antibacterial and immune regulatory effects in human (3). Four meta-analyses have been completed on the use of probiotics for the prevention of AAD in the general population and the results favored probiotics co-administration with antibiotics (2). Probiotics commonly administered in randomized controlled trials of AAD are*: Lactobacillus acidophilus, Lactobacillus bulgaris, Lactobacillus casei, Lactobacillus rhamnosus, Bifidobacteria bifidum, Bifidobacteria longum, Streptococcus thermophiles, Saccharomyces boulardii*. Safety does not appear to be a concern in healthy individuals, although serious infections (e.g., pneumonia, bacteremia, endocarditis, deep abdominal abscesses and meningitis) have been reported in neonates, severely debilitated and/or immuno-compromised individuals.

Probiotic lactic acid bacteria from fermented foods (yogurt, curd, soybean, bamboo shoot and sorghum-based products) have been well documented (9). Phenotypic identification and technological attributes of native lactic acid bacteria present in fermented bamboo shoot products from North-East India have already been analysed (10). The aim of the current study is to evaluate potential therapeutic application of probiotics isolated from Soibum, a fermented bamboo shoot product of Manipur.

MATERIALS & METHODS

Screening of soibum for potential *Lactobacillus* strains

Traditional fermented foods obtained from the households of Imphal region of Manipur are stored under 4°C for the screening of the lactic acid bacteria. The sample was homogenized using buffer in a sterile mortar and pestle. LAB strains were isolated by serial dilution (10^{-1}-10^{-7}) and pour plated on MRS agar plates. The plates were incubated at 37°C for 48 hours under anaerobic conditions. The well grown, discrete colonies were enumerated for total colony forming unit, and sub-cultured strains were isolated at -20°C [9]. Subculture strains were then mass cultured in MRS broth for 24 hours and then subjected to filtration using Whatman filter paper to get cell free supernatant (CSF).

In vitro analyses of the *Lactobacillus* isolate to control antibiotic associated diarrhea

Well diffusion method

Antimicrobial activity is one of the important tests for the assessment of probiotics. Neutralized cell-free supernatant (CFS) was used to test the production of antimicrobial compounds by well diffusion method. Nine different pathogenic bacteria were used as the indicator strains including *Escherichia coli*-MTCC 7416, *Pseudomonas aeruginosa*-MTCC 7903, *Vibrio cholera* and *Enterobacter aerogenes*-MTCC 7325. 18 hours old cultures of indicator strains on the Muller Hinton agar (Hi Media) plates. CFS of the isolates (100 µl) were added to the wells and allowed to diffuse and incubated at 37°C for 24 hours. The diameter (mm) of the inhibition zone was measured to determine the antimicrobial activity [9].

Simple plating assay

The proposed assay utilizes the modified version of the antibiotic disc diffusion assay ("Kirby-Bauer" assay). The Mueller- Hinton agar plates were prepared containing aqueous bacterial csf (final concentration of extract was 1ml/ml of agar). Next, 20 ml of the overnight grown culture of *E. aerogenes, Eescherichia coli, Pseudomonas aeroginosa, Vibrio cholera,* was poured on Mueller-Hinton agar and left for 5 minutes. The excess bacterial cultures were aspirated, and plates were left in the biosafety hood to dry. Next, filter paper discs impregnated with

different antibiotics (Methicillin, Amoxycillin, Vancomycin, Bacitracin, Erythromycin, Penicillin and Kanamycin) were placed on the agar surface and plates were incubated at 37°C overnight. Any change in the diameter of the inhibition zone was measured to determine bacterial sensitivity as per Clinical Laboratory Standards Institute. Any increase in the diameter of the inhibition zone was investigated to determine whether a specific antibiotic has the potential to target the multiple drug resistant bacteria. For controls, *E. aerogenes, Eescherichia coli, Pseudomonas aeroginosa, Vibrio cholera* were swabbed on Mueller-Hilton agar plates containing extract but without antibiotic discs. In some experiments, *E. aerogenes, Eescherichia coli, Pseudomonas aeroginosa, Vibrio cholera* were swabbed on Mueller-Hinton agar plates without extract but with antibiotic discs [16].

Agar overlay method

This method is a modified form of the Agar overlay antimicrobial assay [6]. Lab strain L9 was grown for 48 hours. MRS agar media was poured on petriplate and solidified. Smear inoculation was done with L9 strain. Target pathogens were grown to stationary phase and mixed with 0.6% nutrient agar at 45°C at a concentration of 1ml of target culture per 20ml of agar. The solidified MRS media inoculated with the LSB strain was overlaid with 3ml of the target mixture and incubated overnight at 37°C for 24 hours.

Evaluation of probiotic potential of the selected *Lactobacillus* strain with anti-aad activity

Acidic pH tolerance

The pH tolerance of the strain was tested. Phosphate buffer saline (PBS) solution was prepared for acid tolerance studies by adjusting the pH to 1, 3, 5, 7, and 9 using 1M HCl. 1ml of the test LAB sample was inoculated into these five test tubes containing 9ml of PBS solution and incubated for 24 hours. After incubation, OD was taken at 540nm using spectrophotometer [9].

Bile salt tolerance

To estimate bile tolerance of LAB, each strain was harvested by centrifugation (7000xg for 10minutes), washed twice and PBS, inoculated (1%) into MRS broth containing 0.3%, 0.5%, 0.7% and 1% bovine bile, and incubated at 37°C for 24 hours. Then, the number of viable LAB cells was determined by taking OD using spectrophotometer [13].

Antibiotic susceptibility Test

Antibiotic susceptibility of the isolate L9 was tested against eight selected antibiotics using Himedia anitibiotic discs Vancomycin (VA)-30mg, Bacitracin (B)-10 U, Kanamycin (K)-30mcg, Amoxycillin (AMC)-30mcg, Penicillin G (P)- 10 U and Erythromycin (ERY)-15mcg by Kriby-Bauer Disc Diffusion method. Test LAB of 24-hours culture was inoculated onto MRS agar plates by swabbing using sterile cotton swabs. Antibiotic discs were placed on the inoculated plates. After incubation at 37°C for 24 hours, inhibition zones around the discs were measured and susceptibility was compared to the reference chart of zone size interpretative chart for antibiotics as per CLSI [17].

Cell surface hydrophobicity

In vitro cell hydrophobicity was determined by microbial adhesion to hydrocarbons (MAHC) assay [Collado MC et al., 2008]. Briefly, overnight cultures of the isolates were centrifuged at 60000xg for 10 minutes using cold centrifuge (Eppendorf) and resuspended in 50mM PBS buffer. 1ml of n-Hexane was added to 3ml of bacterial suspension (Ao) and vortexed for 2 minutes. The tubes were kept undisturbed for 30 minutes at 37°C to separate the two phases. Aqueous layer (A) was removed, and OD was measured at 600nm using spectrophotometer (Thermo Scientific). Hydrophobicity was calculated as the percentage in the decrease in the OD of the initial aqueous bacterial suspension due to cells partitioning into a hydrocarbon layer. The percentage of cell surface hydrophobicity (%H) of the strain adhering to n-Hexane was calculated using the equation. %H= [(1 –A)/ Ao) *100] Same procedure was followed for xylene, Ethyl acetate and chloroform. The results were compared with the standard culture of *Lactobacillus plantarum* MTCC 9483 [9].

Resistance to artificial duodenum juice

To evaluate the resistance of the isolates to stimulated gastric juice, 1ml of the overnight culture was suspended in 9ml of the artificial duodenum juice (NaCl-1.28g, KCl-0.239g, $NaHCO_3$-6.4g, Bile slats-0.3%, pepsin-0.1g, sterile distilled water-1L and pH-2.5) and incubated at 37°C for the 0hr, 1hr 2hr and 3hr. Sodium phosphate buffer was used as control. After incubation, viable cell count was measured as CFU ml^{-1} formed by serial dilution and plate count method [9].

Biochemical and molecular characterization of *Lactobacillus* strain with anti- aad activity

Biochemical and morphological characters were studied as per the standard protocol mentioned in reference 9. The isolates were tested for Gram staining, catalase test, lactose hydrolysis, bile salt hydrolysis, carbohydrate fermentation test with different carbohydrates viz. glucose, L-arabinose, maltose, raffinose, D-xylose and arginine. All the isolates were tested with different sugars. To different and respectively marked wells, 1% of sugars

(arabinose, arginine, glucose, maltose, raffinose, and xylose) and bromothymol blue were added. The media was poured to the wells of the microtitre plate and added with 10µl of the isolate was added. After incubation for overnight, the plate was observed for colour change from blue to yellow. Those microorganisms which produced acid in open and closed tubes are described as fermentive [13].

Gram staining

It is very useful staining technique to identify bacteria. In this technique, the fixed smear of bacteria is subjected to four different reagents in the order listed: Crystal violet for 30 seconds; washed with alcohol and applied with safranin for 30 seconds. The washed and dried slide is then observed under microscope. The bacteria which maintained the blue colour of the primary stain is called gram-positive and those colour stained by safranin are called gram negative [32].

Catalase test

Clean and grease free slides were taken and then 2-3 drops of 24 hr culture of the isolate were placed on the slides. 3-4 drops of H_2O_2 were placed on above of the isolate. A catalase positive culture will produce bubbles of oxygen within one minute after addition of H_2O_2 (32). This test is the preliminary confirmatory test for Lactic acid bacteria.

Carbohydrate fermentation

Organisms use carbohydrate differently depending upon their enzyme compliment. The pattern of fermentation is characteristic of certain species, genera, or groups or organisms and for this reason, this property has been extensively used as a method for biochemical differentiation of microbes. MRS broth was prepared in different tubes and marked with different sugars (1% of arginine, arabinose, glucose, maltose, raffinose and xylose) added. Sterilization was done for 15 minutes at 121°C. Then added with bromo cresol purple dye (5ml/ 20ml of MRS broth). The media (190µl) was then poured into the wells using micropipette and L9 CFS (10µl) was added to each well. 24 hours incubation was done, and fermentation is indicated as colour change from blue to yellow. Lactose hydrolysis test is done using the same protocol as that of carbohydrate fermentation test, but with different concentration of lactose (0.5%, 1% and 25%). This test is done to check the capacity of the isolate to treat for lactose intolerance.

Tolerance to different concentration of NaCl

This experiment deals with testing the degree of inhibition of microorganisms that results in with media containing different concentrations of sodium chloride.

To investigate survival of LAB strains under different NaCl concentrations, the strain of LAB was harvested by centrifugation (10,000xg for 10 minutes), washed twice in PBS, inoculated (1%) into MRS broth that had been adjusted to different NaCl concentrations (1, 3, 5, 7 and 9) were incubated at 37°C for 24 hr. After incubation, OD is taken at 540nm using spectrophotometer [13].

Molecular characterization of the selected *Lactobacillus* strain with anti-aad activity

DNA extraction

The isolates were grown in 5ml MRS broth in a rotary shaker at 37°C overnight. The DNA was extracted by conventional method and confirmed with nanodrop technique.

PCR amplification

The DNA was purified and amplified using primer 27 F (AGAGTTTGATCMTGGCTCAG) and 519 R (GWATTACCGCGGCKGCTC). PCR amplification reactions were carried out in a 25µl reaction mixture. 2µl of the DNA was amplified with 2.5µl of 109 PCR buffer, 2.5 µl of 25mM $MgCl_2$, 2.0µl of 2mM dNTPs, 1.0ml of 20pmol primer 27F, 1ML of 20pmol primer 519 R, 0.125µl of LA Taq and water upto 13.875µl. PCR conditions were as follows: initial denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing for 30seconds at 53°C, extension at 74°C for 2 minutes, followed by a final extension at 74°C for 13 minutes. Amplified product was confirmed by agarose gel (1%) electrophoresis and documented using Syngene G-box gel documenting system [Chenappa et al., 2014].

RESULTS AND DISCUSSION

Direct plating & serial dilution

A total of 9 bacterial strains isolated from the fermented bamboo- shoot (Fig.1) were analysed in the present study. The colonies which change the pH of the media to acidic (yellow) were preliminarily screened (Fig 2 and Fig 3). Growths were observed on MRS agar after 24hrs incubation under anaerobic condition. Out of the isolates, nine dominant cultures were used for further studies. The isolates were named as L1, L2, L3, L4, L5, L6, L7, L8 and L9. The sub cultured strains (Fig. 4) were mass cultured, filtered to get cell free supernatant and subjected to antimicrobial analysis.

Antimicrobial activity

The antimicrobial activity of all the isolates was tested against some of the enteropathogenic bacteria, responsible for AAD. Out of the 9 isolates, the best one, L9 was chosen for further analysis. L9 was found to show zone of inhibition for four test pathogens- *Escherichia coli*- MTCC 7416,

Pseudomonas aeruginosa- MTCC 7903, *Vibrio cholera* and *Enterobacter aerogenes*- MTCC 7325. The zone of inhibition was measured in mm. This activity may be due to the bacteriocin- like inhibitory substances.

Well diffusion method

The antimicrobial activities of all the isolates were tested against enteropathogenic bacteria. Among all the strains, the strain L9 showed good antimicrobial activity against *Escherichia coli*- MTCC 7416, *Pseudomonas aeruginosa* - MTCC 7903, *Vibrio cholerae* and *Enterobacter aerogenes*- MTCC 7325 (Fig. 5). The activity may be due to bacteriocin- like inhibitory substances (BLIS) produced by potential *Lactobacillus* strain. The isolates have significant antimicrobial activity against all the indicator strains. The zone of inhibition was measured in mm (Table 1).

Table 1. Antimicrobial activity of the isolate

Sl. No.	Test Organisms	Zone of inhibition (diameter in mm)
1	*Escherichia coli*	17 ± 0.5
2	*Pseudomonas aeruginosa*	14 ± 0.5
3	*Vibrio cholerae*	14 ± 0.5
4	*Enterobacter aerogenes*	22 ± 0.5

Table 2. Bacterial extract enhanced the efficacy of synthetic antibiotics

Sl	Test organisms	Antibiotics	Zone of inhibition	Zone of inhibition
1	*Pseudomonas aeruginosa*	Erythromycin	0	0
		Methicillin	0	0
		Bacitracin	0	0
		Kanamycin	13±.5	16±.5
		Vancomycin	0	0
		Penicillin	0	0
2	*Enterobacter aerogenes*	Erythromycin	24±.5	26±.5
		Methicillin	0	22±.5
		Bacitracin	12±.5	12±.5
		Kanamycin	23±.5	30±.5
		Vancomycin	20±.5	26±.5
		Penicillin	9±.5	30±.5
3	*Escherichia coli*	Erythromycin	0	30±.5
		Methicillin	0	31±.5
		Bacitracin	15±.5	32±.5
		Kanamycin	0	30±.5
		Vancomycin	0	34.15±.5
		Penicillin	0	15±.5
4	*Vibrio cholerae*	Erythromycin	0	0
		Methicillin	0	0
		Bacitracin	0	0
		Kanamycin	18±.5	23±.5
		Vancomycin	0	0
		Penicillin	0	0

Table 3. Percentage of Cell Surface Hydrophobicity of L9 strain.

	Control	Chloroform	Xylene	Ethylene	Hexane
OD (595nm)	1.254	1.221	1.099	0.320	0.590
Hydrophobicity (H%)	0	12.36	2.69	74.48	52.95

Table 4. Biochemical characteristics of the isolate L9

Tests	*Lactobacillus* isolate L9
Morphology	Rod
Gram staining	+ve
Catalase test	-ve
Lactose intolerance	
0.5%	+ve
1.0%	+ve
2.0%	+ve
Growth at different NaCl concentration	
1.0%	+ve
3.0%	+ve
5.0%	+ve
7.0%	+ve
9.0%	+ve
Carbohydrate fermentation	
Glucose	+ve
Arginine	+ve
Maltose	+ve
Arabinose	+ve
Xylose	+ve
Raffinose	+ve

Fig. 1. Fermented Bamboo Shoots

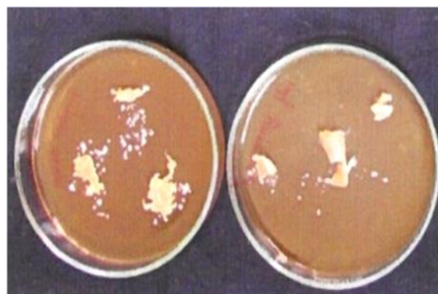

Fig.2.Direct Plating in MRS Agar

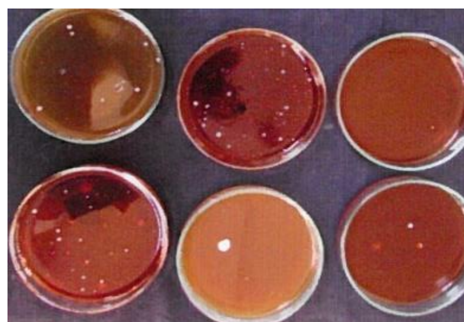

Fig. 3. Serial Dilution Plating on MRS agar

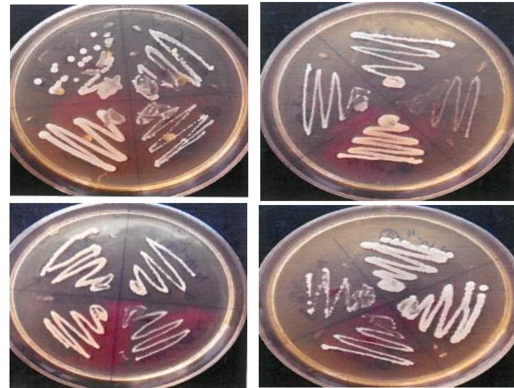

Fig. 4. Subcultures of the *Lactobacillus* isolates.

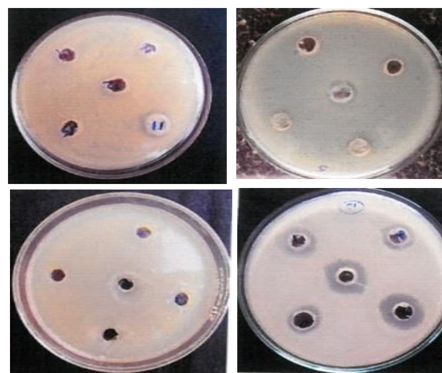

Fig.5. Antimicrobial activity of the isolate L9 against the test pathogens- *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholera* and *Enterobacter aerogenes* (clockwise from top left)

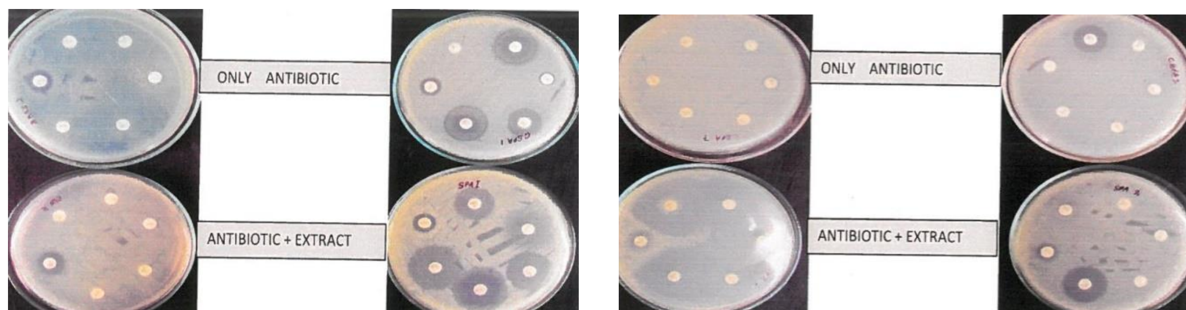

Fig.6. Simple plating antimicrobial assay showing bigger zone of inhibition in plates with L9 extract. *Pseudomonas aeruginosa* (left). *Enterobacter aerogenes* C) *Escherichia coli* D) *Vibrio cholera*

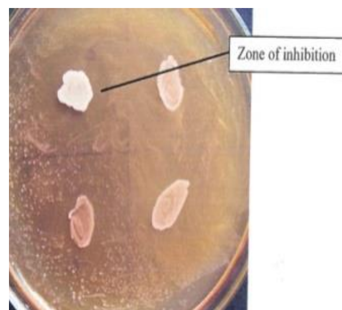

Fig.7. Agar overlay method of L9 showed antimicrobial activity against Enterobacter aerogenes

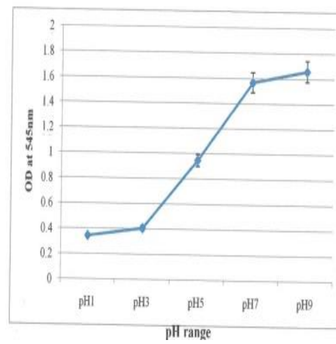

Fig.8. Tolerance of the isolate L9 to different pH (1, 3, 5, 7 & 9)

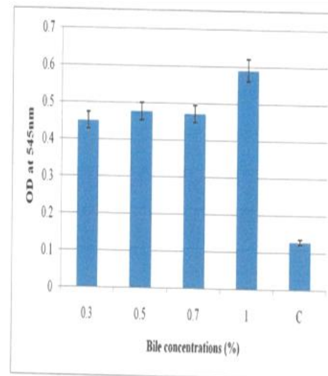

Fig.9. Tolerance of the isolate L9 to different bile salt concentration (0.3%, 0.5%, 0.7%, 1% & Control C

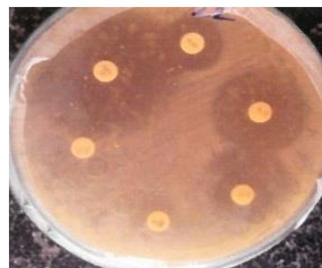

Fig.10. L9 was found to be resistant against the antibiotics- Vancomycin and Kanamycin

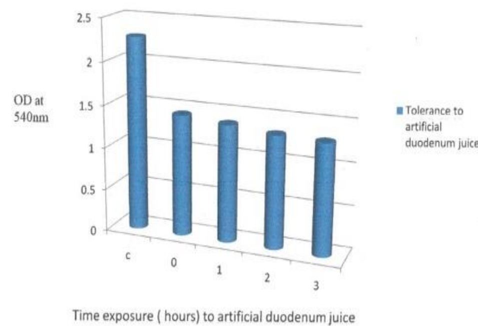

Fig.11. Tolerance of L9 strain to artificial duodenum juice after 0, 1, 2 and 3hr of incubation

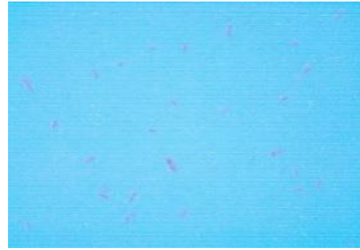

Fig.12. The isolate L9 was found to be Gram positive and rod shape

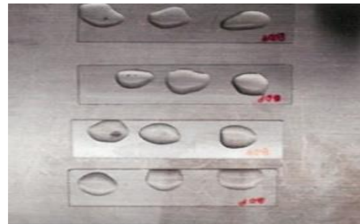

Fig.13. The isolate L9 was found to be catalase negative

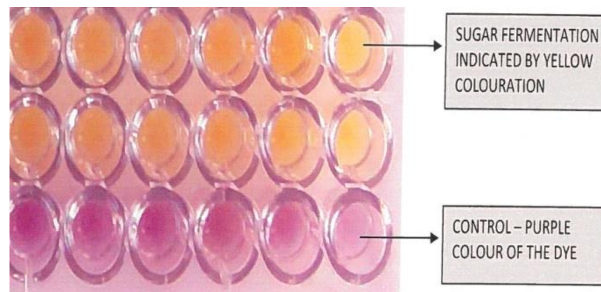

Fig.14. The isolate was found to ferment all the six sugars- arabinose, arginine, glucose, maltose, raffinose, xylose

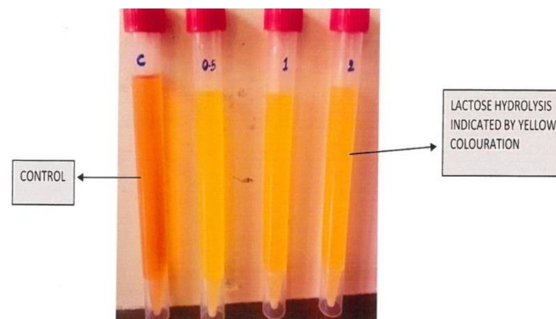

Fig.15. The isolate was found to hydrolyse lactose with different concentrations

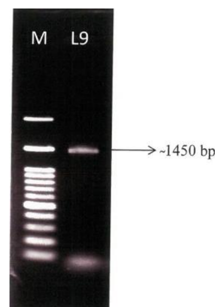

Fig.16. Amplified product confirmed by agarose gel electrophoresis (M-Marker, L9- Isolate)

Simple plating assay

This assay has an advantage of screening a pure compound or natural product against five to eight different antibiotics on the same plate. This technique allows them to contribute in the discovery of potential antimicrobials that target drug- resistant pathways in multiple drug- resistant bacteria. The basis of the proposed assay is that natural products with potential antibacterial activity overcome the resistance phenotype of multiple drug- resistant (MDR) bacteria and render them susceptible to the available antibiotics. The proposed assay has the potential to test the efficacy of various antibiotics on a single plate in an easy to use manner.

In addition, the proposed assay is cost-effective and can be performed in a basic microbiology laboratory. Bacteria employ a variety of strategies to develop resistance against a single group of antibiotics. These include drug- degrading and modifying enzymes, reduced membrane permeability for drugs, expression of drug efflux pumps, overproduction and alteration of drug targets, and by passing the inhibitory pathway. Thus, strategies (or use of natural products) that can bock the afore-mentioned drug resistant mechanisms will allow the clinical use of currently available antibiotics. This is not a novel concept and several studies have reported the use of multiple compounds to produce synergistic or addictive effects against microbial pathogens [18, 19]. One such example is coamoxiclav, a combination of amoxicillin and clavulanic acid. This combination is also effective against amoxicillin- resistant bacteria because clavulanic acid is an inhibitor of β-lactamase, an enzyme which degrades β- lactum drugs including amoxicillin. Other similar combinations which are in clinical use include ampicillin/ sulbactum and piperacillin/tazobactum [20]. The findings reported here suggest that bacterial extract can extend the clinical use of available antibiotics. However, the mechanisms of action remain unknown, and future studies are needed to identify the substance(s) present in the bacterial extract. This assay has an advantage of screening a pure compound or natural product against five to eight different antibiotics on the same plate. Because of its cost- effectiveness and simplicity, this assay could be used by labs which have just basic microbiology set up and limited funding and allow pathways in multiple drug- resistant bacteria, the mechanisms of which will be explored in further studies.

The bacterial extract of L9 enhances the efficacy of tested antibiotics against the pathogens. The zones of inhibition (Fig.6) were found to be bigger in plates added with L9 extract and the measurement of the zones of inhibition are tabulated (Table 2).

Overlay assay

The target bacteria distributed through the top agar layer grew to produce a homogenously turbid lawn. The L9 strain smear also grew well and inhibition of bacterial growth caused a reduction in the turbidity in the lawn near the agent: the greater the antibacterial action, the wider the zone of inhibition (Fig. 7). Thus, the antibacterial strength of the agent may be judged by the width of the zone of inhibition around it.

Acidic pH and bile tolerance

Tolerance to acidic pH is one of the pre-requisites for the validation of probiotics. The strains L9 showed increased tolerance to pH 2 for 12hrs of incubation, when compared to control 12hrs incubation. The viability of strain decreased as the incubation time increases with decrease in pH of the media. At pH 3 for 1-2 hrs of incubation, the strain showed minimal reduction and did not find any significant differences in viable cell count. Before reaching the gastrointestinal tract, probiotic bacteria must first survive transit through the stomach and have their health promoting effects as metabolically viable active cells when they arrive in the colon [22]. Therefore, the various LAB isolated form fermented bamboo shoot were screened for their acid and bile resistance. The L9 strain was resistant at acidic condition, after incubation for 2hrs at pH 1 and stable in 1% bovine bile for 24hrs (Fig. 8 and 9 respectively). Accordingly, the strain could most likely survive in the stomach and the small intestine and colonize in the large intestine.

Antibiotic susceptibility test

Antibiotics are major tool utilized by the medical and pharmacological industries to fight pathogenic bacteria; however, antibiotic resistance can cause significant danger and suffering for many people with common pathogen infections, and is a growing problem that complicates the treatment of important nosocomial and community-acquired infections [23]. In order to be used as probiotics, LAB that exhibit profitable effects on the health of the host must show an ability to resist various antibiotics. Many studies on the antibiotic sensitivity and resistance of LAB, which were isolated from various products and human or animal gastrointestinal tracts, have been reported, the lactobacilli strains isolated from infant feces were resistant to Kanamycin and streptomycin, but affected by amoxicillin, chloramphenicol, erythromycin, penicillin G and tetracycline [31]. Therefore, the antibiotics resistance of acid and bile-resistant LAB

was assessed using Lim & Im protocol, with a little modification. L9 strain was highly resistant at higher than 30 µg/ml concentrations of vancomycin, kanamycin [Fig. 10].

Cell surface hydrophobicity

Cell surface hydrophobicity has been conducted to know the ability of LAB strains to adhere the gut region, as it is one of the criteria to be a potential probiotic strain. Microbial adhesion to hydrocarbon assay (MAHC) was conducted to know the *in vitro* cell surface hydrophobicity of the *Lactobacillus* strain isolated in the present study. The result of MAHC assay was that the isolate L9 strain showed optimum cell surface hydrophobicity (74.48%) with ethylene and minimum (2.69%) with xylene (Table 3). Our results are in correlation with the results obtained by Collado et al. [26], where 44.2% of maximum adhesion and 25.06% of minimum adhesion was reported by *Lactobacillus plantarum* species (26).

Resistance to artificial duodenum juice

Tolerance to artificial duodenum juice is one of the criteria for the evaluation of survival potential of probiotic *Lactobacillus* strains under stimulated gastric conditions. The lactic acid bacteria strain in the present study showed the tolerance to artificial duodenum juice. It is observed that the tolerance of the LAB strains correlates/ inversely proportional with the incubation time. When the incubation time increased from 0 to 3 hrs, the CFU ml^{-1} of the LAB strain has been decreased. The strain exhibited maximum tolerance to stimulated gastric juice. Several reports on the survival ability of *Lactobacillus* under stimulated gastric juice have been documented supporting the present study [16, 17]. The *Lactobacillus* strain of the present study could resist the stimulated gastric conditions. The L9 strain was significantly affected by the artificial duodenum juice for 1 and 2 hr incubation. However, there was a decrease in the viability of the LAB strain after 3hr incubation and showed reduction in their viable count of 1.4 CFU ml^{-1}, respectively after 3hr incubation (Fig.11).

Biochemical characterisation of LAB

The isolate was found to Gram positive (Fig.12), Catalase negative (Fig. 13) and were considered as presumptive LAB strain. Biochemical characterization reveals that the isolates were homo fermentative. It has the ability to ferment sugars (Fig.14) - glucose, maltose, arginine, arabinose, xylose, raffinose and lactose [34]. The isolate may also be used for treating lactose intolerance as it showed Lactose hydrolyses (Fig.15) for different concentration of lactose. *Lactobacillus* strain isolated from other fermented foods, such as fermented broccoli stems “Yan- tsai-shin” [21] showed similar kinds of reports. The optimum growth was seen at 1% NaCl (Table 4).

Molecular identification of the selected isolate L9

PCR amplification was carried out using 27 F and 1492 R primers and the amplicon size was approximately 1450 bp (Fig. 16). Amplified product was confirmed by agarose gel (15%) electrophoresis and documented using Syngene G-box gel documenting system [9,29]. The 16s rRNA sequence profiling is one of the cost-effective tools and enables the researchers to identify the complex microbial communities upto species level at exceptional depth and resolution.

CONCLUSION

Antibiotic treatment may disturb the colonization resistance of gastrointestinal flora, resulting in a range of clinical symptoms, most notably, diarrhoea. This is due to overgrowth of many enteropathogens like *Clostridium difficile, E. aerogenes, E. coli, P. aeroginosa, V. cholerae.* Using of probiotics from traditional fermented foods will be beneficial to treat AAD as using of antibiotics cause abnormal colonization of microflora inside the gastrointestinal tract and may lead to antibiotic resistant microorganisms. Probiotics have been administered both prophylactically and therapeutically in an attempt to modify the mucosal, epithelial, intestinal and systematic immune activity. They benefit human health and improve microbial balance in the intestinal tract and display both antibacterial and immune regulatory effects in humans. The isolate L9 was found to be effective against the enteropathogens. The efficacies of commercially available antibiotics were also found to be increased when incorporated along with the isolate. This technique of using the isolate along with the antibiotics could be useful in the treatment of antibiotic resistant pathogens.

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